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Inhibition of NF-κB, iNOS mRNA, COX2 mRNA, and COX catalytic activity by phenyl-*N*-*tert*-butylnitrone (PBN)

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Abstract

Previously, the spin trapping agent phenyl-*N*-*tert*-butylnitrone (PBN) has been shown to decrease the level of nitric oxide synthase mRNA in vivo. This inhibition is suggested to be an underlying mechanism for PBN's wide variety of pharmacological actions in animal models. However, the determination of PBN's cellular pharmacological activities has not been carried out, but is necessary for the understanding of the effects in vivo. Since the known pharmacological effects of PBN are primarily anti-inflammatory in nature, in this study we determined the inhibitory activities of PBN against two inflammatory factors: inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX2). We show here that PBN decreases steady state COX2 mRNA level and COX2 catalytic activity in macrophage cell culture at supra-pharmacological concentrations. While PBN decreases iNOS mRNA, it does not inhibit iNOS catalytic activity, which is consistent with previous in vivo studies. We also studied nuclear factor κB (NF-κB), a transcription factor that can rapidly activate the expression of genes involved in inflammatory, immune and acute phase responses. The binding of NF-κB to iNOS gene has been shown to be critical for iNOS gene expression, and the promoter region of COX2 gene contains NF-κB consensus sequence. We show that PBN inhibits lipopolysaccharide-mediated increase of NF-κB DNA binding activity with a lower concentration than that for the non-steroidal anti-inflammatory drug (NSAID), salicylate. Furthermore, we show that PBN inhibits COX2 catalytic activity, suggesting that PBN has an NSAID-like function.

Keywords: Phenyl-*N*-*tert*-butylnitrone; Inducible nitric oxide synthase; Inducible cyclooxygenase; Nuclear factor κB; Nitric oxide; Prostaglandin E_2; Nitrone; Spin trap; Free radical; Macrophage

1. Introduction

The spin trapping agent phenyl-*N*-*tert*-butylnitrone (PBN) has been shown to prevent death after endotoxin shock in rodents when administered with a lethal dose of endotoxin [1–5]. In the same event, PBN has been shown to significantly reduce the formation of nitric oxide in the liver [6,7]. This reduction has been shown to be associated with the decrease of steady-state nitric oxide synthase (iNOS) mRNA level [7]. In animal models, other pharmacological activities of PBN include neuro-protection [8–11], anti-aging [12–14], and anti-diabetic effect [15], suggesting that the effect of PBN is largely anti-inflammatory. Although these pharmacological

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effects are diverse, PBN's inhibition against iNOS mRNA synthesis is suggested to be the common underlying mechanism. But the relationship between PBN's inhibition of iNOS mRNA and its free radical trapping capabilities is not understood.

Macrophages are considered to play a major role in endotoxin shock [16]. When activated by endotoxin, macrophages elicit the formation of inflammatory cytokines, which then activate macrophages and other cells to promote iNOS gene induction. Although nitric oxide is necessary for physiological activities, the highly expressed iNOS produces large amounts of nitric oxide in cells and tissues in an uncontrolled manner, resulting in a catastrophic breakdown of various physiological functions. PBN's interaction with macrophages appears important in this event, but the specific pharmacological activity of PBN in isolated cells is not well characterized. In contrast, cellular anti-inflammatory functions of other antioxidants such as salicylate, *N*-acetyl cysteine and L-lipoic acid have been extensively investigated [17–23]. Salicylate [17–19], *N*-acetyl cysteine [20,21] and L-lipoic acid [22] inhibited nuclear factor κB (NF-κB) and iNOS mRNA in cells but with relatively high concentrations as compared to therapeutic doses. However, it is clear that the determination of cellular pharmacological activities of these drugs is necessary for the understanding of pharmacological activities in vivo. In the present study, using isolated macrophages, we determined the inhibitory activities of PBN against the two major inflammatory enzymes iNOS and inducible cyclooxygenase (COX2) by quantifying produced nitrite and prostaglandin E_2 (PGE_2), respectively. PBN was also tested for the alteration of iNOS mRNA and COX2 mRNA levels, and the catalytic activities of these two enzymes.

A major transcription factor for the iNOS gene is NF-κB [24], a redox-sensitive transcription factor that is activated by oxidants and inhibited by antioxidants [23]. We hypothesized that PBN may exert an antioxidant effect which prevents NF-κB from binding to DNA, thus decreasing iNOS mRNA level. In order to verify this hypothesis, we tested PBN for inhibition of lipopolysaccharide-mediated NF-κB binding activity in macrophages.

2. Materials and methods

2.1. Materials

PBN was synthesized in these laboratories and purified with vacuum sublimation. PBN was also obtained from commercial sources such as Aldrich Chemical Co. (Milwaukee, WI). Reagents for nitrite assay (Griess reaction) were obtained from Fisher Scientific (Pittsburgh, PA). LPS (from *Escherichia coli*) and fetal calf serum (FCS) were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. Sources of other reagents are shown where the name of the reagent first appears.

2.2. Macrophage isolation and activation

Primary peritoneal macrophages [25] were obtained from male Balb/c mice (6–8 weeks old, from Charles-River, Indianapolis, IN), 5 days after intraperitoneal injection of eliciting reagent, 4% brewer's thioglycollate medium (1.5 ml, Defco, Detroit, MI). After euthanasia, the peritoneal cavity was infused with 5 ml ice-cold RPMI 1640 medium (with glutamine, Fisher), which was subsequently collected. The obtained cell suspension was washed with RPMI 1640 medium twice. Cells were re-suspended in conditioned RPMI 1640 medium containing 5% FCS, 100 μg/ml streptomycin (Sigma), and 100 units/ml penicillin (Sigma), and seeded onto 24-well cell culture plates (Fisher). Cells were incubated for 2 h at 37°C in a 5% CO_2 incubator to promote adherence to the bottom of the well. These cells were then incubated in conditioned RPMI 1640 medium containing LPS (50 ng/ml, from *E. coli*, serotype 0127:B8, Sigma) and interferon γ (IFN-γ, 250 U/ml) in the absence or presence of PBN (1 to 10 mM). Incubation lasted 5 h for Northern analysis and 15 h for immunoblotting, and 1 h for NF-κB determination.

Cell viability after incubation with 1, 5, and 10 mM PBN for 15 h in conditioned RPMI 1640 medium was determined using LDH release assay (Sigma). LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR) was also used to access the cytotoxicity of PBN. The LIVE (viability) method is based on the measurement of intracellular esterase

activity through calcein AM fluorescence and the DEAD (cytotoxicity) method measures plasma membrane integrity through fluorescence from ethidium homeodimer. These assays were performed three times using cells obtained from separate animals.

2.3. Determination of nitrite formation

Nitrite, the oxidation product of NO, was measured by the Griess reaction [26]. Cells were activated with LPS (50 ng/ml) and IFN-γ (250 U/ml) in the absence or presence of PBN. After incubation for 15 h, supernatants were discarded and replaced with the fresh conditioned RPMI 1640 medium followed by 3 h incubation. iNOS expression was measured by means of the nitrite amount in the medium. The medium from incubated cells was mixed with an equal volume of the Griess reagent (Fisher) in a well of a 96-well cell culture plate. The absorption at 540 nm was recorded with a micro-plate reader (Thermomax, Molecular Device, Palo Alto, CA), and the absorption coefficient was calibrated using a sodium nitrite solution standard (Fisher).

For the iNOS catalytic activity measurement, cells were incubated for 15 h with LPS (50 ng/ml) and IFN-γ (250 U/ml) to fully express iNOS. Supernatant was discarded and fresh medium containing PBN was added, followed by 3 h incubation. Nitrite in this medium was quantified with the Griess reaction. Each experiment was performed in triplicate and repeated three times using cells obtained from separate animals.

2.4. Determination of PGE_2 production

PGE_2 produced from activated macrophages was quantified using an enzyme immunoassay (EIA) kit for PGE_2 (Cayman Chemical, Ann Arbor, MI). Cells were activated by LPS (50 ng/ml) and IFN-γ (250 U/ml) to express COX2 in the presence or absence of PBN. Cells were washed twice, and the substrate (30 μM sodium arachidonate, Sigma) was added in conditioned RPMI 1640 medium. After 0.5 h incubation, the produced PGE_2 in the medium was quantified to determine COX2 expression. These media were diluted and transferred to a PGE_2 antibody-coated 96-well culture plate in the EIA kit and treated according to manufacturer's instruction. Absorbance at 410 nm was recorded using a microplate reader.

For the COX2 catalytic activity measurement, COX2 in cells was fully expressed by the incubation with LPS (50 ng/ml) and IFN-γ (250 U/ml) for 15 h. Subsequently, cells were washed and added with arachidonate (30 μM), followed by 0.5 h incubation in the presence of PBN. The produced PGE_2 in the medium was quantified to determine COX2 catalytic activity. Each experiment was performed in triplicate and repeated three times using cells obtained from separate animals.

2.5. Northern blotting

mRNA for iNOS and COX2 in macrophages was determined by Northern blotting. Mouse peritoneal macrophages were incubated with LPS (50 ng/ml) and IFN-γ (250 U/ml) with or without PBN for 5 h. At this incubation time it was confirmed that the expression of the mRNA was maximized (data not shown). Total RNA was isolated from macrophages (3 million cells) using TRIzol (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. Equivalent amounts of total RNA (typically 20 μg) were loaded onto a 1% formaldehyde–agarose (Fisher) gel. After electrophoresis, RNA was transferred overnight to a nylon membrane (MSI, Westboro, MA) by capillary action and secured to the membrane by UV cross-linking. After soaking the membrane in the hybridization buffer at 65°C for 30 min, hybridization (65°C) was performed overnight with ^{32}P-labeled cDNA for mouse iNOS (ALEXIS, San Diego, CA) or ^{32}P-labeled oligonucleotide probe for COX2 (Molecular Biology Resource Facility, University of Oklahoma Health Science Center, Oklahoma City, OK). ^{32}P-Labeled probe for the ribosomal RNA 28s (Santa Cruz Biotechnology) was used to confirm equal loading of mRNA. The blot was then washed twice and exposed to X-ray film (Fuji) at −80°C for 15 h. Densities of the blots were determined using Eagle Eye II still video system (Stratagene, La Jolla, CA). Northern blotting experiments were conducted three times using cells obtained from separate animals.

2.6. Immunoblotting

Relative amounts of iNOS and COX2 protein expressed in macrophages were quantified using immunoblotting. Cells were incubated for 15 h with LPS (50 ng/ml), IFN-γ (250 U/ml), and with or without PBN at 37°C. The protein expression in cells leveled in 15 h incubation (data not shown). Subsequently, the boiling cell-lysis buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol) was added to the well and cells were disrupted in the medium. The lysate was centrifuged at $14\,000 \times g$ for 30 min and the supernatant was subjected to electrophoresis. Total protein in the supernatant was determined using Lowry's method (Total protein assay kit, Sigma) and the same amount of protein was loaded to each lane of the gel. Electrophoresis was performed on an SDS–PAGE (7.5–12% gradient). Separated protein bands were transferred and treated with rabbit anti-mouse macrophage iNOS or COX2 antibody (0.1 μg/ml, Transduction Lab), and then treated with horseradish peroxidase-conjugated IgG antibody (Amersham). The bands were detected by the ECL system (Amersham) with a typical exposure time of 15 s. Densities of immunoblots were quantified using the same system as used in Northern blotting. Immunoblotting was performed three times using cells obtained from separate animals.

2.7. Electrophoretic mobility shift assay (EMSA)

The binding activity of NF-κB in nuclear extracts of LPS/IFN-γ-stimulated macrophages to its consensus oligonucleotide was evaluated by means of EMSA, using a non-radioactive EMSA kit, DIG Gel Shift Kit available from Boehringer-Mannheim (Indianapolis, IN). Briefly, cells were incubated with LPS (50 ng/ml), IFN-γ (250 U/ml), and with or without PBN for 1 h. Nuclear extracts were prepared as described by Dignam et al. [27]. The NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C3′, Santa Cruz Biotechnology, Santa Cruz, CA) was end-labeled with digoxigenin (DIG) using terminal transferase. Nuclear extracts (10 μg) were added to DIG-labeled NF-κB oligonucleotide (0.8 ng) in a buffer containing 2 μg of poly(dI-dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol (total volume of 20 μl). This solution was subjected to electrophoresis on 5% non-denaturing polyacrylamide gel, followed by electro-transfer to nylon membrane. The non-labeled NF-κB-specific oligonucleotide was used as a competitive probe to confirm the specific binding. Furthermore, a mutant probe (Santa Cruz Biotechnology) was used to demonstrate protein binding specificity. The membrane was subsequently treated with anti-DIG-antibody, which then treated with alkali phosphatase-conjugated IgG. The chemiluminescent substrate CSPD (Boehringer-Mannheim) was added and the resulting chemiluminescence was detected by autoradiography. The band densities were quantified using the same system as used in Northern blotting. EMSA was performed twice using cells obtained from separate animals.

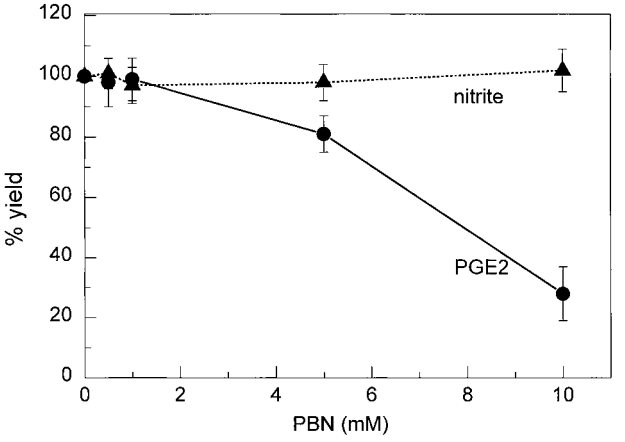

Fig. 1. Inhibition of iNOS and COX2 catalytic activity by PBN as determined by nitrite and PGE_2 production. Nitrite (broken line) and PGE_2 (solid line) were quantified as a measure of catalytic activity of iNOS and COX2, respectively. Mouse peritoneal macrophages were incubated with LPS (50 ng/ml) plus IFN-γ (250 U/ml) for 15 h to fully express iNOS and COX2. For nitrite assay, fresh medium with or without PBN was added and incubated for 3 h. Griess reagent was added to supernatant to quantify nitrite spectrophotometrically. In PGE_2 determination, the activated cells were incubated with the medium containing sodium arachidonate (30 μM) with or without PBN. Amounts of PGE_2 in supernatant were quantified with enzyme immunoassay. Points in the figure show the percent amount relative to the amount obtained in PBN-free system. Error bars denote S.D. calculated from the data obtained from triplicate measurement from three separate animals (average of total of nine data points).

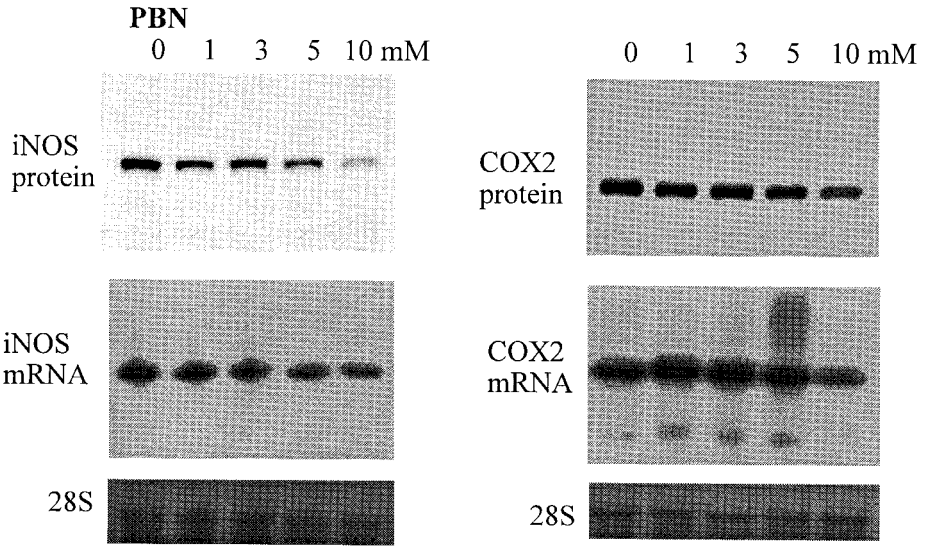

Fig. 2. Inhibition of iNOS and COX2 by PBN as determined with immunoblotting and Northern blotting. Mouse peritoneal macrophages were activated with LPS and IFN-γ for 15 h in the presence of the indicated concentrations of PBN. Whole cell protein was subjected to SDS–PAGE analysis. Bands for iNOS (131 kDa) and COX2 (70 kDa) were detected using monoclonal antibody for iNOS and COX2 (top panel). For Northern blotting, cells were activated with LPS and IFN-γ for 5 h in the presence of the indicated concentrations of PBN. Whole cell RNA was subjected to electrophoretic analysis with agarose gel. The separated bands were hybridized with the radiolabeled iNOS or COX2 probe and detected (bottom panel). Fluorograms indicated as 28S are from 28S ribosomal RNA, whose densities indicate the loaded amount of RNA to the gel. Densities of these bands were determined to estimate percent inhibition.

3. Results

3.1. PBN's effect on cell viability

Cell viability after incubation with PBN for 15 h was measured with LDH release assay ($n=3$). The results were 96 ± 6% (PBN 1 mM), 92 ± 7% (PBN 5 mM), and 81 ± 10% (PBN 10 mM). The results from LIVE/DEAD assay were 96 ± 2% live/2 ± 1% dead (PBN 1 mM), 94 ± 2% live/8 ± 5% dead (PBN 5 mM), and 82 ± 3% live/11 ± 4% dead (PBN 10 mM).

3.2. PBN's effect on iNOS and COX2 catalytic activities

Experiments were conducted to determine whether PBN inhibits iNOS and COX2 catalytic activity. Macrophages which had been incubated with LPS plus IFN-γ for 15 h were used as the source of iNOS and COX2. The amounts of nitrite and PGE_2 produced from macrophages in the presence of PBN were plotted in Fig. 1. This shows that PBN is an inhibitor of COX2 catalytic activity with IC_{50} (concentration at 50% inhibition) approximately 8 mM. PBN is not an inhibitor of iNOS catalytic activity.

3.3. Inhibition of iNOS and COX2 protein expression

Expression of iNOS and COX2 protein was evaluated in macrophages which had been activated with LPS and IFN-γ in the presence of PBN for 15 h. Immunoblotting of iNOS and COX2 protein indicated that PBN reduces these proteins in a dose-dependent manner (Fig. 2, top panel). These results are consistent with those obtained by nitrite assay.

3.4. Inhibition of iNOS mRNA and COX2 mRNA

The expression of iNOS and COX2 mRNA was determined to show that PBN alters steady-state concentrations of iNOS and COX2 mRNA. Northern blot analysis of macrophages treated with PBN showed suppression of both iNOS mRNA and COX2 mRNA (Fig. 2, bottom panel) in a dose-dependent manner. The densities of those bands were normalized with respect to corresponding 28S bands,

and are plotted in Fig. 4. This graph assumes the density in the absence of PBN as 100%.

3.5. Effect of PBN on NF-κB binding

DNA binding activity of NF-κB in nuclear extracts in LPS/IFN-γ-stimulated macrophages was evaluated by means of EMSA. The amount of NF-κB that bound to the NF-κB-specific oligonucleotide increased in stimulated cells (Fig. 3, lanes 2 and 6) as compared to unstimulated cells (Fig. 3, lane 1). The band disappeared in the presence of excess non-labeled (cold competitive) NF-κB-specific oligonucleotide (Fig. 3, lane 7). Furthermore, the NF-κB band from the sample added with a mutant probe showed minor change (Fig. 3, lane 8). These two facts support the authenticity of the NF-κB band. Nuclear extracts obtained from PBN-treated cells exhibited a reduced amount of NF-κB-oligonucleotide complex (Fig. 3, lanes 3–5), indicating that PBN inhibited NF-κB in a concentration-dependent manner.

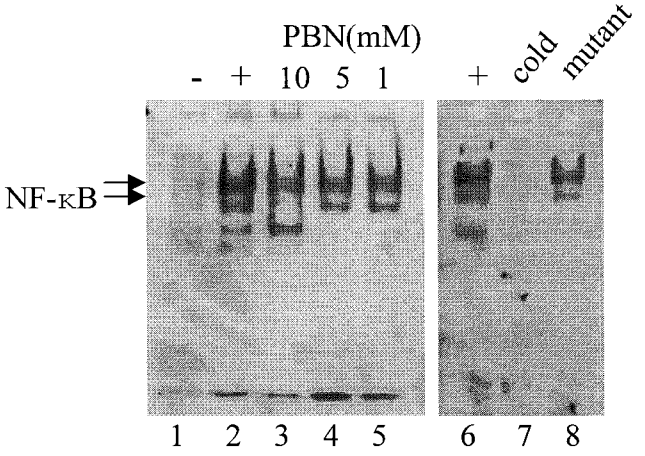

Fig. 3. Inhibition of NF-κB by PBN as determined by fluorescence-detected electrophoretic mobility shift assay (EMSA). Mouse peritoneal macrophages were stimulated with LPS and IFN-γ for 1 h in the presence of indicated concentrations of PBN. Nuclear extracts from these cells were obtained and combined with a labeled NF-κB-probe nucleotide. NF-κB was quantified from the density of the retarded bands (double bands marked as NF-κB) on the gel. Lane 1, (−) non-stimulated; lane 2, (+) stimulated; lane 3, stimulated in the presence of 10 mM PBN; lane 4, stimulated in the presence of 5 mM PBN; lane 5, (−) stimulated in the presence of 1 mM PBN; lane 6, (+) stimulated; lane 7, cold – an excess amount of non-labeled NF-κB probe added (competition assay); lane 8, mutant – an excess amount of mutant NF-κB probe added (specificity assay).

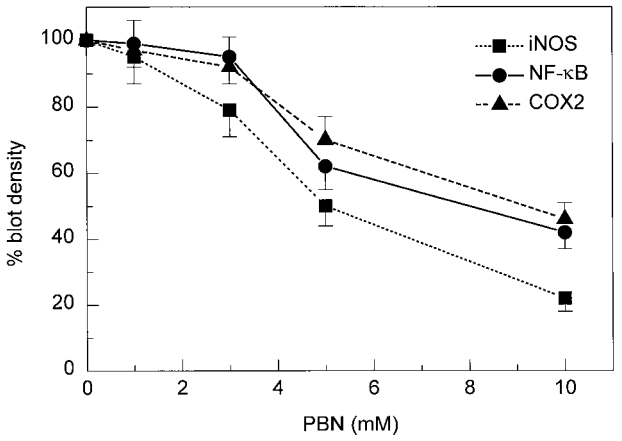

Fig. 4. Inhibition of NF-κB, iNOS mRNA, and COX2 mRNA by PBN as illustrated by the reduction of the blot density. NF-κB in the macrophage nucleus was quantified by fluorescence-detected EMSA as described in Section 2, and the percent density of NF-κB band as compared to the control (no PBN) band was plotted as a function of PBN concentration (solid line). mRNAs of iNOS and COX2 were quantified by Northern blotting as described in Fig. 2. The relative band density was quantified and plotted as a function of PBN concentration (dotted line and dashed line).

The decrease of the band density strongly suggests that the amount of NF-κB that has DNA binding activity was decreased by the presence of PBN. IC_{50} of PBN for NF-κB inhibition was estimated to be approximately 8 mM. Relative densities of NF-κB bands were plotted in Fig. 4.

4. Discussion

In these experiments, we investigated the cellular pharmacological activities of PBN using mouse peritoneal macrophages in order to obtain a basic knowledge to understand PBN's in vivo activities. The cytotoxicity of PBN was low even at 10 mM, suggesting that the present inhibition activities were not caused by the toxicity of PBN. In macrophages, iNOS mRNA was decreased in the presence of PBN to one-half at approximately 6 mM. Although PBN inhibited iNOS mRNA in vivo; however, in mice, PBN's ID_{50} for iNOS mRNA in the liver was approximately 1 mmol/kg [7]. If one assumes that animal body is simulated by a water bag, this number may be translated into 1 mM PBN which is homogeneously distributed in the body. It is not surprising

to see such large discrepancies in doses between in vivo and cellular systems [17–19]. Although the reason for such discrepancies is not well understood, in the case of PBN, preferential distribution of the drug to the lipid-rich organ, such as the liver, may partly account for this discrepancy. PBN is a lipophilic agent with octanol/water partition ratio of 15:1 [28].

PBN inhibited both COX2 mRNA expression and COX2 catalytic activity. This means PBN can reduce PGE_2 in two ways: by decreasing COX2 expression and inhibiting COX2 activity, suggesting that PBN could be classified as an NSAID (non-steroidal anti-inflammatory drug), because NSAIDs' major pharmacological action is by the COX inhibition [29]. Also, PBN decreased the formation of nitrite and PGE_2 (Fig. 1) due to the decrease of iNOS/COX2 protein, which is shown by Western blotting results. This decrease in proteins must be the result from the decrease in iNOS/COX2 mRNA. These results indicate that PBN inhibits iNOS and COX2 at the transcription or pre-transcription stage. In addition, evidence is increasing that many inflammatory events are associated with co-existence of active iNOS and COX2 [30–33]. NO and PGE_2 appear to enhance the inflammatory action of each other. Indeed, co-inhibition of iNOS and COX2 by two independent inhibitors has been shown to be very effective in the carageenan-induced edema model in rats [32]. It is possible that PBN's pharmacological action is enhanced by the co-reduction of NO and PGE_2. However, the role of COX2 inhibition in PBN's pharmacological action in vivo remains to be elucidated.

There is a clear inhibition of NF-κB by PBN in LPS/IFN-γ-activated cells (Fig. 3). IC_{50} for the NF-κB inhibition was estimated to be approximately 8 mM and is consistent with that of iNOS mRNA inhibition, suggesting that NF-κB plays a major role in the transcription of the iNOS gene [24]. It has been shown that the promoter (5′-flanking) region of COX2 gene also contains two separate NF-κB consensus sequences [34]. Similar IC_{50}s for COX2 mRNA (10 mM) and NF-κB (8 mM) suggest the importance of NF-κB in COX2 gene transcription [35]. It should be noted that sodium salicylate and aspirin have shown to inhibit NF-κB in cells at relatively higher concentrations such as $IC_{50} = 15$ mM [17].

NF-κB is a redox-sensitive transcription factor and has been shown to be activated by pro-oxidants such as hydrogen peroxide, and NF-κB is also inhibited by many antioxidants [23,36–38]. In resting cells, NF-κB exists in the cytosol as a complex with the endogenous inhibitor IκB. It was proposed that oxygen radicals directly destroy IκB, activating NF-κB and promoting its translocation to the nucleus and the subsequent trans-activation. The question to be asked is whether PBN acts like a radical scavenger which directly protects IκB from destruction. Such simple scenarios do not seem to hold because of the recent finding of IκB kinase (IKK) [39–42]. IκB destruction is shown to be preceded by its phosphorylation by IKK, followed by ubiquitination and proteolysis. Moreover, other kinases have also been shown to respond to oxidative stress to elevate their activities, which could contribute to the destruction pathway. It is possible that antioxidants like PBN act as radical scavengers to keep these kinases from activation.

Acknowledgements

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References

[1] G. Novelli, P. Angiolini, G. Consales, R. Lippi, R. Tani, in: G.P. Novelli, F. Ursini (Eds.), Oxygen Free Radicals in Shock, Karger, Basel, 1986, pp. 119–124..
[2] K. McKechnie, B.L. Furman, J.R. Parratt, Circ. Shock 19 (1986) 429–439.
[3] S.A. Hamburger, P.B. McCay, Circ. Shock 29 (1989) 329–334.
[4] H.W. Pogrebniak, M.J. Merino, S.M. Hahn, J.B. Mitchell, H.J. Pass, Surgery 112 (1992) 130–139.
[5] J.F. French, C.G. Thomas, T.R. Downs, D.F. Ohlweiler, A.A. Carr, R.C. Dage, Circ. Shock 43 (1994) 130–136.
[6] T. Miyajima, Y. Kotake, Biochem. Biophys. Res. Commun. 215 (1995) 114–121.
[7] T. Miyajima, Y. Kotake, Free Radic. Biol. Med. 22 (1997) 463–470.

[8] J.W. Phillis, C. Clough-Helfman, Neurosci. Lett. 116 (1990) 315–319.
[9] J.M. Carney, R.A. Floyd, J. Mol. Neurosci. 3 (1991) 47–57.
[10] X. Cao, J.W. Phillis, Brain Res. 644 (1994) 267–272.
[11] J. Folbergrová, Q. Zhao, K. Katsura, B.K. Siesjö, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 5057–5061.
[12] J.M. Carney, P.E. Stark-Reed, C.N. Oliver, R.W. Landrum, M.S. Cheng, J.F. Wu, R.A. Floyd, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3633–3636.
[13] R.A. Floyd, Science 254 (1991) 1597.
[14] R. Edamtsu, A. Mori, L. Packer, Biochem. Biophys. Res. Commun. 211 (1995) 847–849.
[15] T. Tabatabaie, Y. Kotake, G. Wallis, J.M. Jacob, R.A. Floyd, FEBS Lett. 407 (1997) 148–152.
[16] C. Szabó, New Horizons 3 (1995) 2–32.
[17] E. Kopp, S. Ghosh, Science 265 (1994) 956–959.
[18] E.E. Aeberhard, S.A. Henderson, N.S. Arabolos, J.M. Griscavage, F.E. Castro, C.T. Barrett, L.J. Ignarro, Biochem. Biophys. Res. Commun. 208 (1995) 1053–1059.
[19] A.R. Amin, P. Vyas, M. Attur, J. Leszczynska-Piziak, I. Pate, G. Weissmann, S.B. Abrahamson, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7926–7930.
[20] M. Shibanuma, T. Kuroki, K. Nose, FEBS Lett. 353 (1994) 62–66.
[21] K. Pahan, F.G. Sheikh, A.M. Namboodiri, I. Singh, Free Radic. Biol. Med. 24 (1998) 39–48.
[22] Y.J. Suzuki, M. Mizuno, H.J. Tritschler, L. Packer, Biochem. Mol. Biol. Int. 36 (1995) 241–246.
[23] R. Schreck, K. Albermann, P.A. Baeuerle, Free Radic. Res. Commun. 17 (1992) 231–237.
[24] Q.W. Xie, Y. Kashiwabara, C. Nathan, J. Biol. Chem. 269 (1994) 4705–4708.
[25] A.H. Ding, C. Nathan, D. Stuehr, J. Immounol. 41 (1988) 2407–2412.
[26] L.C. Green, D.A. Wanger, J. Glogowski, P.L. Skipper, J.S. Wishnok, R. Tannenbaum, Anal. Biochem. 126 (1982) 131–138.
[27] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Nucleic Acids Res. 11 (1983) 1475–1489.
[28] E.G. Janzen, M.S. West, Y. Kotake, C.M. DuBose, J. Biochem. Biophys. Methods 32 (1996) 183–190.
[29] J.R. Vane, J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxyall, D.A. Willoghby, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2046–2050.
[30] K.K. Wu, Adv. Pharm. 33 (1995) 179–207.
[31] J.A. Corbett, G. Kwon, J. Turk, M.L. McDaniel, Biochemistry 32 (1993) 13767–13770.
[32] D. Salvemini, P.T. Manning, B.S. Zweifel, K. Seibert, J. Connor, M.G. Currie, P. Needleman, J.L. Masferrer, J. Clin. Invest. 96 (1995) 301–308.
[33] D. Salvemini, T.P. Misko, J.L. Masferrer, K. Seibert, M.G. Currie, P. Needlemen, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 7240–7244.
[34] S.B. Appleby, A. Ristimaki, K. Neilson, K. Narko, T. Hla, Biochem. J. 302 (1994) 723–727.
[35] J.F. Schmedtje Jr., Y.S. Ji, W. Liu, R.N. SuBois, M.S. Runge, J. Biol. Chem. 272 (1997) 601–608.
[36] R. Schreck, P. Rieber, P.A. Baeuerle, EMBO J. 10 (1991) 2247–2258.
[37] P.A. Baeuerle, T. Henkel, Annu. Rev. Immunol. 12 (1994) 141–179.
[38] P.J. Barnes, M. Karin, N. Engl. J. Med. 336 (1997) 1066–1071.
[39] J.A. DiDonato, M. Hayakawa, D.M. Rothwart, E. Zandi, M. Karin, Nature 388 (1997) 548–554.
[40] F. Mercurio, H. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J.W. Li, D.B. Young, M. Barbosa, M. Mann, A. Manning, A. Rao, Science 278 (1997) 860–866.
[41] J.D. Woronicz, X. Gao, Z. Cao, M. Rothe, D.V. Goeddel, Science 278 (1997) 866–869.
[42] E. Zandi, D.M. Rothwart, M. Delhase, M. Hayakawa, M. Karin, Cell 91 (1997) 243–252.